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Heterotopic Tracheal Transplants: Techniques and Applications

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Heterotopic tracheal transplants, placed subcutaneously in syngeneic rats have been extensively used in our laboratory. The objective of these experiments was to study the toxic and/or carcinogenic effect of several compounds on the respiratory tract mucosa. This was attained by exposing the transplants to an intraluminal pettet containing the toxicant or carcinogen mixed with an adequate matrix (gelatin, beeswax, stearyl alcohol, silastic, etc.). By varying the concentration of the test chemicals, it is possible to study dose-response relationships, and by changing the pellet matrix, the effects of release rate (dose rate) can be analyzed. Several end points can be studied, such as histological changes in the mucociliary epithelium, changes in mucus secretion, tumor induction and changes in the *in vitro* behavior of the epithelial cells after *in vivo* exposure. In addition, by de-epithelializing the tracheal transplants and reseeding them with another cell population, e.g., from previously treated cell cultures or from human specimens and transplanting them subcutaneously in nude mice, completely new vistas on the effect of chemicals can be opened.

Introduction

The direct application of toxicants and/or carcinogens to the respiratory tract epithelium by inhalation exposure, intratracheal injections or pellet implantation into the bronchus or lung has been frequently employed in toxicology and carcinogenesis studies (*1–3*). Although each of these techniques offers some advantages, with the first two it is often difficult to determine the precise dose delivered to each segment of exposed airway mucosa. Furthermore, multiple exposures are usually required to obtain the desired effect, and several portions of the respiratory tract may be exposed to different doses of the test substance. The pellet implantation technique obviates these problems by exposing a circumscribed area of respiratory mucosa to a known quantity of test substance which diffuses out of the pellet, either into the bronchus or into the lung. This technique, on the other hand, is complicated and often impairs the normal respiratory function of the treated lung, not infrequently leading to premature death of the animals. A model, which has the advantages of the *in loco* pellet implantation technique and does not jeopardize the respiratory functions of the experimental animal, was developed in our laboratory by transplanting subcutaneously tracheas obtained from syngeneic rats (*4,5*). Previous experiments by other investigators had shown that lung parenchyma as well as tracheas could be transplanted and exposed to carcinogens (*6–9*). Some of these early studies were successful in producing carcinomas after methylcholanthrene or dibenzanthracene exposure, and papillomas after diethylnitrosamine exposure. The approach used in our laboratory is to transplant tracheas subcutaneously and expose them only after the grafts have been fully established and revascularized. The use of several types of pellet-matrices has permitted the controlled release of varied total doses of test substances at a predetermined dose rate. The combined use of fully established and revascularized transplants and slow release pellet matrices has eliminated to a great extent, hyperacute toxic damage to the graft and surrounding tissues, as well as decreased the incidence of peritracheal sarcomas, which have been previously reported (*9*).

Techniques

Since most of our own work, as well as the majority of the experiments published to date, has been carried out in rats, the following description of the transplantation techniques will deal mainly with the procedures using this species. Differences or variations in techniques when using other species will be mentioned when appropriate.

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Donor Tracheas

Ten- to twelve-week-old inbred specific pathogen-free Fischer 344 rats are used as donors for tracheas. A wide anterior thoracotomy is performed under anaesthesia with either methoxyfluorane or sodium pentobarbital (Fig. 1). After exposure of the anterior mediastinum under strict aseptic conditions, the trachea is separated from the surrounding tissues, and a polyethylene tubing (length 20 mm, outer diameter 5 mm) is sutured to one side of the trachea to preserve the original length of the organ. If this step is omitted, the trachea contracts to approximately one-half of its original length and tends to curl. The trachea is severed below the first tracheal ring and immediately above the carina. The laryngeal end of the trachea is tied up with a silk suture. The trachea is removed from the donor animal and placed in a Petri dish containing Hank's balanced salt solution with streptomycin and penicillin. The tracheas can be kept in this solution for several hours without any apparent loss in viability. Before transplantation, the other extreme of the trachea is closed with a silk suture. This step can be omitted without noticeable differences because after transplantation the open end will be closed by granulation tissue.

Recipient Animals

Rats of the same strain and age are used as recipients. They are anesthetized with methoxyfluorane, their backs are shaved with electric clippers and cleansed with 5% peracetic acid solution in water, and an incision, 1 to 2 cm long near the midline, is made on the dorsum, parallel to the vertebral column between the scapulae. With blunt dissection, two subcutaneous pouches, one at each side of the incision, are carefully prepared to lodge the tracheal transplants, together with their supporting polyethylene tubing (Fig. 2). In order to standardize the position of the tracheal transplant (tt), the transplant is so placed that the distal end points toward the head of the recipient animal. The wound is then closed with surgical clips and the animal with one tracheal transplant at each side of the dorsal midline is returned to its cage. For practical purposes, usually no more than two tracheas are transplanted per animal, but in some cases, more transplants per animal can be used with little inconvenience (*10*). This is not advisable if during a long-term study the tracheal transplants are likely to develop either tumors or extensive fibrosis, situations which interfere with the usually nonsynchronous processes taking place in the neighboring tracheas.

Similar techniques of syngeneic tracheal transplants have also been applied with another rat strain (*10*) and other species, i.e., hamsters (*5,11,12*), dogs (*13–15*), and mice (*5,16*). After transplantation, the tracheal transplants become revascularized in approximately 5 days. During the first 2 days, the respiratory epithelium becomes necrobiotic, and most cells slough off (*5*). Remaining basal cells rapidly repopulate the epithelium, which is completely reconstituted and normal after 2

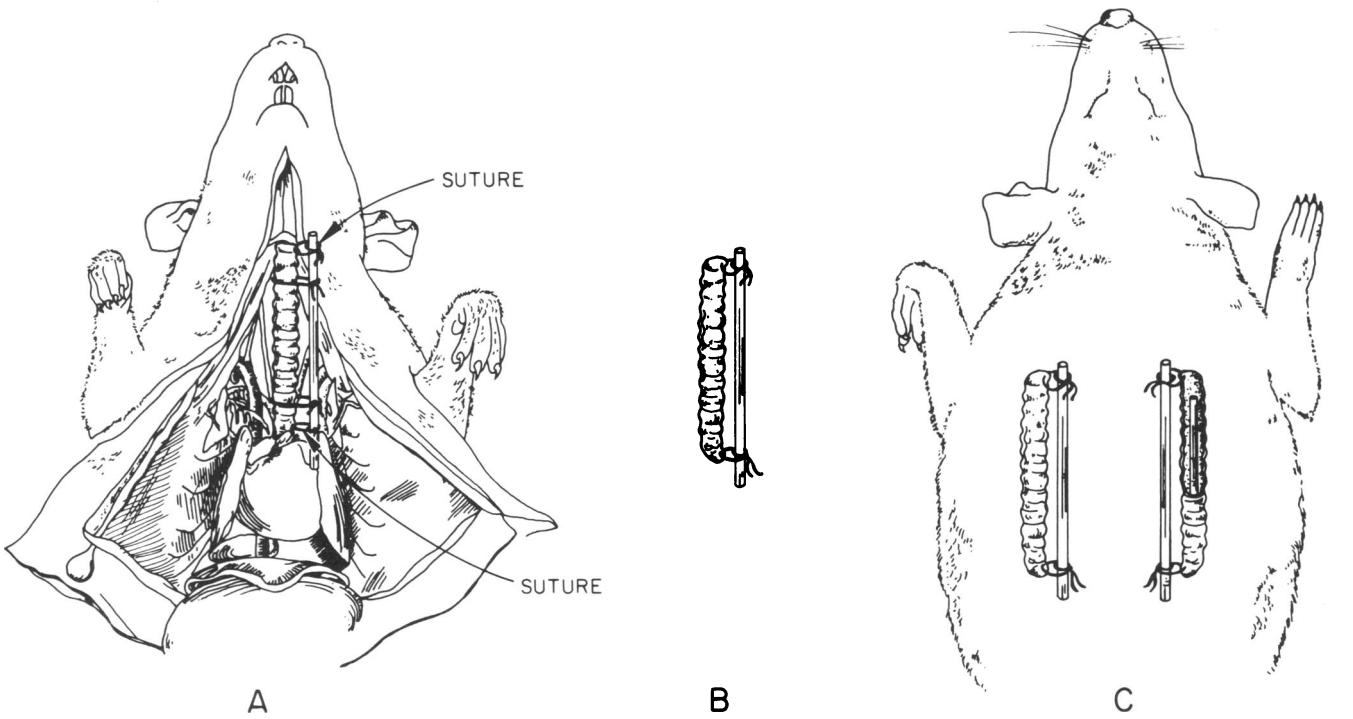

FIGURE 1. Transplantation procedure: (*A*) the wall of the donor trachea is sutured to a polyethylene tubing (arrows), and both ends are tied up by another pair of sutures; (*B*) the trachea and the tubing are excised and (*C*) transplanted in the dorsal subcutaneous tissue of a syngeneic recipient.

FIGURE 2. Tracheal transplant in the subcutaneous tissue of the rat dorsum. The skin has been resected for better view.

weeks. Between 2 and 4 weeks after transplantation, the tracheal transplant is completely revascularized, and the epithelial lining becomes indistinguishable from that of the host trachea (Fig. 3). It is usually at this time that the tracheal transplant is exposed to chemical agents. The transplant is exposed to carcinogens or toxicants in a controlled and quantitative fashion by inserting the appropriate pellets (see below), containing the test chemical, into the tracheal lumen. This is achieved through a small incision, which can be easily closed by either a simple suture or by using large tantalum hemostatic clips (E. Weck Co.). Likewise, if the exposure is to be terminated before the tracheal transplants are removed, the pellets are extracted through another incision at the cephalic end of the transplant and closed again.

Materials and Vehicles for Intratracheal Pellets

The selection of the pellet vehicles or matrices depends on the nature of the materials to be released from the pellet and on the desired rate of release. In most cases, a long exposure of the tracheal mucosa to constant concentrations of chemicals and in consequence a slow release of the test chemical from the pellet is desired. Tracheal transplants can be exposed to two types of test agents which require different vehicles and pellet manufacturing techniques: lipophilic substances, such as polycyclic aromatic hydrocarbons (PAH) and 12-O-tetradecanoyl-phorbol 13-acetate (TPA), which are incorporated into nondegradable matrices such as beeswax (*17*), silastic (*18*) or lycra fibers (*12*) and poorly soluble particles or fibers, such as nickel subsulfide, asbestos or arsenic trioxide embedded into degradable vehicles such as gelatin. This latter approach implies relatively short exposure times and an abrupt release of the test agent since gelatin dissolves rapidly at body temperature. Alternative matrices for poorly soluble particles and fibers which enable longer exposure times are under development.

Technology of Pellet Production

The rate of release of the test agent from the pellets depends mainly on the nature of the pellet matrix. Materials for pellet matrices must meet the following criteria: should be nontoxic; should not interact with the carcinogen/toxicant; should be easily fabricated into solid cylindrical pellets (14 mm × 1.4 mm diameter for rat trachea) at low temperatures to avoid any decomposition of the test chemical. Release from such monolithic cylindrical pellets containing the dispersed test agent is controlled by the rate of diffusion of the chemical through the pellet matrix to the boundary surface as proposed by Higuchi (*19*). Invariably, an initial high release rate (burst effect) occurs (*20*) until the equilibrium is reached and the release takes place in a controlled fashion.

Lipid Matrices. Beeswax, stearyl alcohol and cholesterol have been employed in our laboratory for slow release of PAH and TPA. Relatively low-melting (up to 150°C) matrices containing the dissolved/dispersed

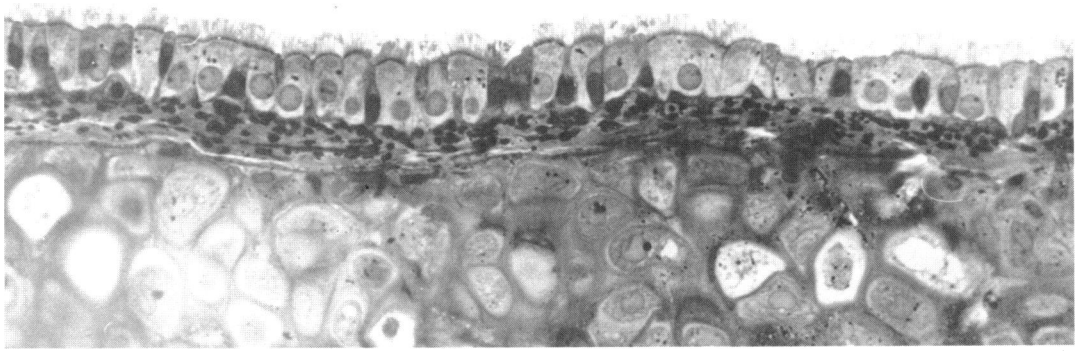

FIGURE 3. Normal mucociliary epithelium lining the tracheal lumen 7 weeks after transplantation. (Toluidine blue, Epon, × 360.)

agent can be fabricated most conveniently in the pellet maker (*4*) (Fig. 4). The test agent and lipid substance is put into a glass scintillation vial and placed in the recess of an aluminum block which is heated on a thermostatically controlled electric heater. The block should be drilled for accommodating a thermometer to monitor the temperature. Heaters (Cole Palmer Instruments) equipped with immersion thermistor probes have been found satisfactory. These probes are placed in another cavity drilled in the block. Occasionally, it is difficult to push the pellets out of the mold by using the push pin. In such a situation, the mold should be reheated by placing in a water bath to a temperature which facilitates extrusion.

The pellet maker works best if the dispersed test agent does not show any tendency to settle. If settling occurs, the matrix is melted in a hot mortar and the agent is mixed with the matrix thoroughly, using the pestle and mortar and allowing it to cool until it solidifies. The mix is scraped out from the mortar and packed into a cylindrical mold of a sodium press (No. 1 Greenerd Arbor Press, Fisher Scientific Co.). This mold is fitted with a die with a 1.5 mm diameter orifice, through which the pellets can be extruded by manually driving a plunger fitted with a rack and pinion mechanism. The mold is warmed to a suitable temperature (below the melting point of the mix) and extruded out quickly. The extruded material is cut into proper lengths by using a channeled Teflon block very much like a carpenter's miter box.

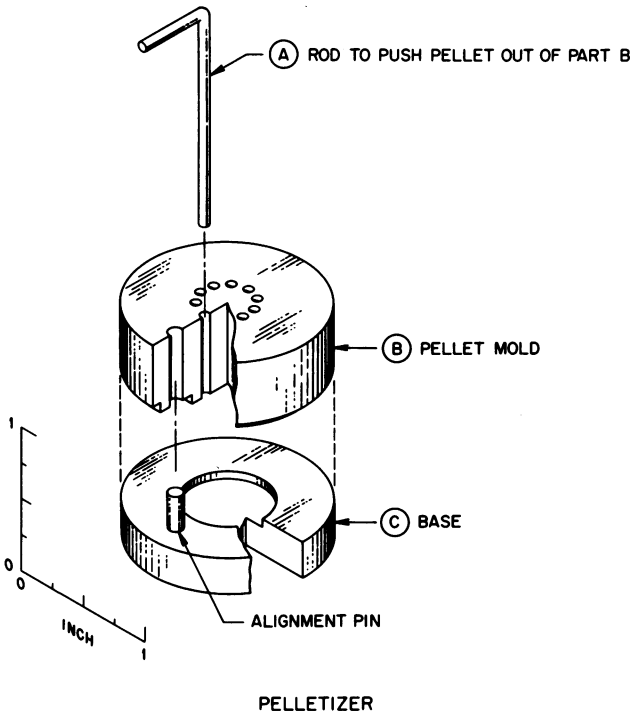

FIGURE 4. Schematic representation of the pellet maker used for manufacturing carcinogen-containing beeswax or gelatin pellets. From Griesemer (*4*).

Pellets Containing PAH. Controlled release of polycyclic hydrocarbons like benzo(a)pyrene (BaP), 7,12-dimethylbenz(a)anthracene (DMBA) from beeswax alone and from cholesterol–beeswax matrices has been reported earlier (*17*). The presence of cholesterol in a beeswax–cholesterol matrix retards the release of PAH in direct proportion to the percentage of cholesterol present. The use of cholesterol of highest purity is essential, and commercially available highest purity cholesterol (Eastman Kodak) should be purified further by the method of Fieser (*21*) and checked for purity by gas chromatography on a 3% SP-2250 column (Supelco).

We have recently found an undesirably high background in the epithelial focus (EF) assay (*22*, M. Terzaghi, unpublished data) as well as the induction of stratified metaplasias after exposing tracheal transplants to cholesterol. In addition, some cholesterol autoxidation products have been found to be mutagenic (*23*). Stearyl alcohol has been found to be an acceptable substitute for cholesterol since it shows very low background in the EF assay. The release rates of DMBA

from beeswax–stearyl alcohol (1:9) and beeswax–cholesterol (1:9) matrices are shown in Figure 5. The use of beeswax is necessary as a binder, since pellets are extremely fragile and difficult to handle when made out of cholesterol or stearyl alcohol alone. A typical preparation of stearyl alcohol–beeswax DMBA pellets is described below.

Weighed amounts of DMBA (Eastman Kodak), purified by crystallization from ethanol as described previously (*17*), and stearyl alcohol, and beeswax (purified by filtration through a steam-heated fritted glass funnel) are heated together in a scintillation vial placed in an aluminum block until a clear melt is formed (65°C). The hot melt is transferred into the well of the pellet maker heated to the same temperature and the matching half is placed on the well, forcing the melt to flow into the holes where, following rapid chilling, cylindrical pellets are formed. A copper block (7.5 cm × 7.5 cm diameter) chilled in ice was found to be very convenient for cooling the pellet maker.

In order to directly assay the amount of DMBA in the pellets, a weighed aliquot of the pellet is dissolved in 10 mL of benzene in a capped scintillation counting vial placed in a shaker water bath at 37°C. The absorbance at 301 nm is read after appropriate dilution, and the concentration of the DMBA determined using a molar extinction coefficient of 78,700. Neither beeswax nor stearyl alcohol or benzene shows any appreciable absorbance at this wavelength. The pellets are best stored in a deep-freeze under nitrogen in the absence of light.

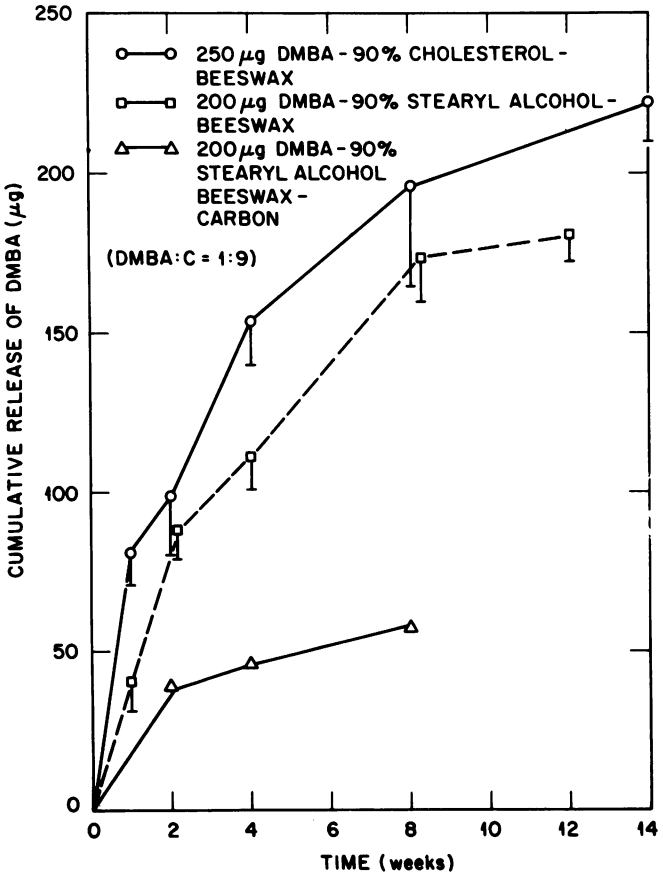

FIGURE 5. Release rates of DMBA from several pellet matrices.

If a slower release rate is desired, DMBA may be absorbed on carbon particles and dispersed in the stearyl alcohol–beeswax (9:1) matrix and made into pellets using the sodium press. Carbon particles (Barnaby Chaney) are passed through sieve #200 and sieve #325 and the particles retained on the latter are used.

DMBA–carbon (1:9) is prepared as follows. Carbon particles (9.0 g) are added with stirring to a hot solution of DMBA (1.1 g) in acetone (160 mL) in a beaker. The acetone is allowed to evaporate in the hood at room temperature. All of the dried, caked mixture from the wall of the beaker is scraped down, and the side of the beaker rinsed with 3 mL acetone. The mixture is heated gently with constant stirring and scraping. After all of the acetone has evaporated, the mixture is heated to about 145°C, with continued scraping and stirring. The mixture becomes dry and powdery. The final product is cooled in a desiccator. To assay the amount of DMBA in the mixture, samples are extracted in a double thickness cellulose thimble (Whatman 1317–403) placed in a Soxhlet apparatus with benzene. Ultraviolet absorption spectrum measurements show that DMBA is not decomposed by this treatment and the absorption of DMBA into carbon is uniform. Weighed amounts of DMBA–carbon are mixed together with stearyl alcohol and beeswax using a hot mortar and pestle, transferred into the mold, heated to 58°C, and extruded into pellets using the sodium press. The uniformity of the pellets can be checked by assaying for DMBA content. A typical *in vivo* release rate of carbon-adsorbed DMBA from these pellets is shown in Figure 5.

Pellets Containing 12–0-Tetradecanoylphorbol 13–Acetate (TPA). Beeswax can be used as a matrix and the TPA pellets can be conveniently made using the pellet maker. Since the molar extinction of TPA is low, it is advisable to use radioassay by incorporating [^{3}H]TPA for quantitative determinations of TPA release. An aliquot can be counted directly in a toluene-based scintillation fluid. Figure 6 shows the release pattern from a beeswax pellet containing 100 μg TPA.

Water-Soluble and Degradable Matrices. Twelve percent aqueous gelatin has been successfully used as a vehicle for rapid release of poorly soluble particles and fibers, such as Ni_3S_2, asbestos, carbon particles and glass fibers. It has also been used for the rapid release (and in consequence for short, acute exposures) of PAH.

Using a warm pestle and mortar, the agent is mixed thoroughly with the gelatin solution and allowed to cool to room temperature while triturating until the mix solidifies. It is then transferred to a scintillation counting vial and heated in an aluminum block to 30°C and

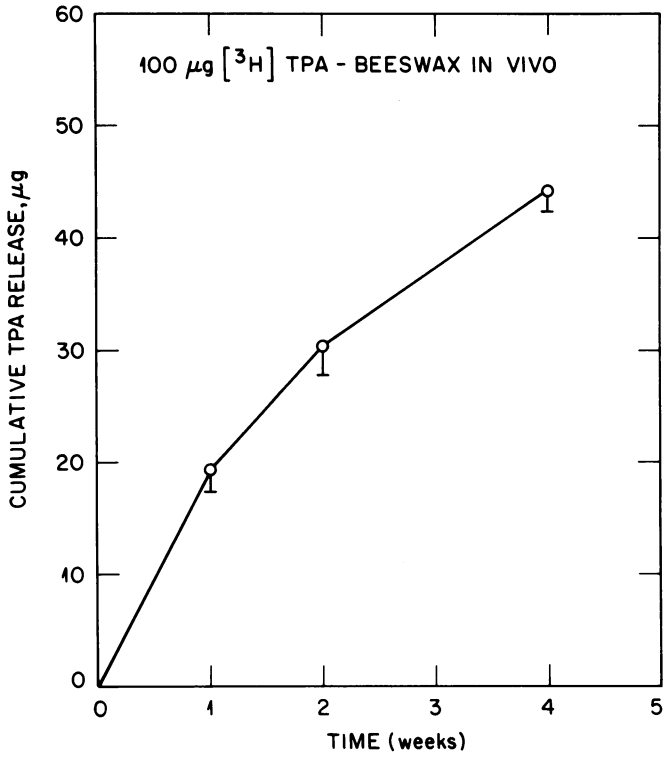

FIGURE 6. Release of TPA from beeswax pellets.

made into pellets using the pellet maker. The pellets are then dried on plastic Petri dishes placed inside a vacuum desiccator over sodium hydroxide pellets. The dried pellets are then assayed for uniformity. In most cases, the pellet is weighed, stirred in water, and filtered through a tared millipore filter. The filter is thoroughly washed with water, dried, and weighed. The concentration of agent in gelatin should not vary more than ± 5%.

Preliminary data suggest that particulate and fibrous material can be released in a continuous slow fashion from stearyl alcohol–beeswax (9:1 ratio), or polyglycolic acid pellets (material used in resorbable surgical sutures; in contact with body fluids it slowly degrades by hydrolysis). These matrices gradually release the embedded particulate material, which then comes in contact with the tracheal epithelium.

Evaluation of Effects

After a short- or long-term exposure to chemicals, simple macroscopic evaluation of the transplanted organ can reveal significant alterations, such as paratracheal or intratracheal cysts or aneurismal dilatations (frequently seen after destruction of part of the wall and subsequent migration of epithelium to an extraluminal position), solid tumors, atrophy and fibrosis, hypertrophy due to accumulation of secretion, etc.

Evaluation of Intratracheal Content. The type of intratracheal contents, as seen by naked eye, can point to the nature of the changes taking place in the epithelium, e.g., keratin can be recognized as semisolid white clumpy masses, and is indicative of squamous metaplasia and/or neoplasia, purulent secretion will signify inflammation and large quantities of mucous secretion will indicate hypertrophy of the secretory epithelium.

Biochemical analyses of the tracheal secretions have characterized the normal mucin fraction of the rat tracheal transplants (*24*). The purified mucin from the transplant contained high molecular weight glycoprotein with a carbohydrate and amino acid content characteristic of mucins. It is well known that similar fractions obtained from tracheal organ cultures undergo changes when exposed to an altered environment such as vitamin A-deficient media (*25*). Changes in the mucin secretion characteristics have also been described in neoplastic epithelial cell lines derived from carcinogen-treated tracheal epithelium (*26*). Mucous secretions from tracheal transplants could serve as an ideal model for biochemical studies of *in vivo* alterations after exposure to toxicants or carcinogens. The potential value of such a nondestructive analysis can not be overstated, and this model offers the possibility of performing long-term studies and obtaining multiple secretion samples from the same transplanted organs. Another type of nondestructive analysis, namely exfoliate cytology, can be performed easily in a longitudinal study of the effects of chemicals on the tracheal epithelium. Although this possibility has not been exploited, all the techniques are available (*27*) and the sampling procedures, which should produce minimum possible damage to the tracheal transplant, could be easily developed.

Histological Study of Tracheal Transplants. Histological observation of tissue sections is the most frequently employed method for evaluating the state of the epithelium and wall of the tracheal transplant. Most studies have relied on routine paraffin embedding, and some have preferred the higher resolution provided by plastic embedding (*28–31*). Several papers have dealt with the ultrastructural characteristics of the normal and altered epithelium (*28,29*).

The most critical issue when studying the distribution and frequency of lesions in the tracheal transplant is the sampling of each trachea. In most studies, the tracheas were cut transversally into rings 1 to 1.5 mm thick. The tracheal pieces are kept in proper order during dehydration and embedding by passing a silk thread through the surrounding connective tissue of each ring. The tissues were embedded maintaining the rings in proper order and orientation. Three to four sections 50 μm apart were then performed, thus further enlarging the sampled area. An alternative method, permitting an ample visualization of the luminal epithelium using only one or two blocks of tissue is

the so-called "Swiss roll," in which the trachea is cut frontally in two pieces, then rolled cephalocaudally, and tied with thread to keep it in this position (*32*). Another method which could permit a selective sampling of tracheal regions has been used for detecting squamous metaplasias of the larynx. This involves staining the whole organ (fixed or unfixed) in either pyronin (*33*) or alcian blue–phloxine (*34*) and then processing for histology only the positively stained areas. This method can be used for the detection of squamous metaplasias in the tracheal transplants, and it seems especially indicated when using tracheas from large animals.

In Vitro *Studies of* in Vivo-*Exposed Cell Populations.* *In vitro* techniques offer new possiblities for the study of sequential changes presumed to occur during the *in vivo* development of neoplasia. It has been shown that shortly after carcinogen exposure of tracheal transplants, epithelial cells which have a markedly increased growth capacity can be isolated from the tracheal mucosa (*22,35*). A series of techniques collectively referred to as the EF assay has been devised to quantitate *in vitro* the emergence of carcinogen-altered and neoplastic cell populations *in vivo* in tracheas exposed to carcinogen. A detailed protocol has been previously described (*22*). Briefly, the EF assay entails the following sequential phases. Heterotopic tracheal transplants are exposed to PAH as described above. At various time intervals after initiation of exposure, beginning prior to the development of frank neoplastic or even before the appearance of preneoplastic lesions, tracheal transplants are removed from host animals. Luminal epithelial cells are dispersed enzymatically and seeded into tissue culture dishes in order to establish primary cultures, the number of EF (proliferating epithelial cell foci) per dish is scored. When isolated, each EF contains roughly 5×10^4 cells. They are removed from the primary dish enzymatically and each EF seeded into a separate secondary culture dish. The fraction of EF which can be subcultured two or more times is noted. All subculturable EF (EF_s) are tested for the capacity to grow in soft agarose. In this system, the capacity to grow in soft agarose has been found to be well correlated with the capacity of a cell population to yield tumors when inoculated IM into immunosuppressed rats (*35,36*).

The EF assay has made it possible to monitor the development *in vivo* of cell compartments endowed with different proliferative and neoplastic potentials *in vitro*. Epithelia from normal noncarcinogen-exposed tracheas yield few EF in primary culture. Epithelia from carcinogen-exposed tracheas yield 10 to 100 times more EF than control cultures. The capacity of EF to be subcultured (EF_s) and the capacity of EF_s to grow in soft agarose (EF_s, ag+) appear to reflect the severity of carcinogen-induced changes in tracheal epithelium. The frequency of EF_s and EF_s, ag+ populations increase both with carcinogen dose and increased time after exposure (*22,37*).

Applications

Most studies employing the tracheal transplant model have dealt with the induction of neoplastic and preneoplastic lesions after *in vivo* exposure to polycyclic aromatic hydrocarbons. This model makes possible other studies in the field of toxicology and carcinogenesis. Some of the most recent applications are listed and briefly described below.

Early Effects of Toxicants and Carcinogens

The aim of these studies is to evaluate the toxic effects of several chemicals on the tracheal tissues (especially the epithelium) over an 8-week period. Most of the chemicals thus far evaluated in our laboratory were known or suspected carcinogens or closely related substances. Topping et al. (*38*) compared the acute effects of seven PAH on the tracheal epithelium by exposing the tracheal transplant with beeswax pellets containing 1 mg of pyrene (P), benzo(e)pyrene (BeP), anthracene (A), benz(a)anthracene (BaA), dibenz(ac)-anthracene (DBacA), benzo(a)pyrene (BaP) or dimethylbenz(a)anthracene (DMBA). The release of PAH from the pellets was determined by ultraviolet spectrophotometry at 3 days, 1, 2, 4 and 8 weeks. All PAHs were released continuously during the 8-week period. Approximately, 1 mg of PAH was released from the pellets containing P, BeP, BaP and A, whereas only approximately 500 μg was released after 8 weeks from the pellets containing the other PAHs. The toxic effects on the respiratory epithelium were evaluated by measuring the percentage of luminal surface occupied by normal, hyperplastic and metaplastic epithelia (Fig. 7). Although all compounds proved to be toxic for the tracheal epithelium, the degree of alterations induced in the tracheal transplants was variable, (i.e., the potent carcinogens (BaP and DMBA) showing a maximum of early toxic effects and the noncarcinogens or weak carcinogens (BeP, A, P) inducing less severe changes or short duration. A similar study was undertaken in order to evaluate the effects of chrysotile A and crocidolite asbestos in gelatin pellets (*39*). Both substances produced intense dose-dependent inflammatory reaction and epithelial alteration over a period of 4 weeks. The effect of crocidolite lasted somewhat longer than that of chrysotile A. Another carcinogen which induced extensive early toxic alterations was nickel subsulfide (*40*). Other particulates have been tested in the same model in our laboratory, i.e., arsenic trioxide, glass fibers, and charcoal particles. All these induced a weak or no response (Klein-Szanto et al., unpublished data). Although there are many complicated and interacting factors involved, (e.g., dose, dose rate, schedule of administration, etc.), these experiments indicate that strong carcinogens are usually very irritant and inflammatogenic for the respiratory tract epithelium, and that

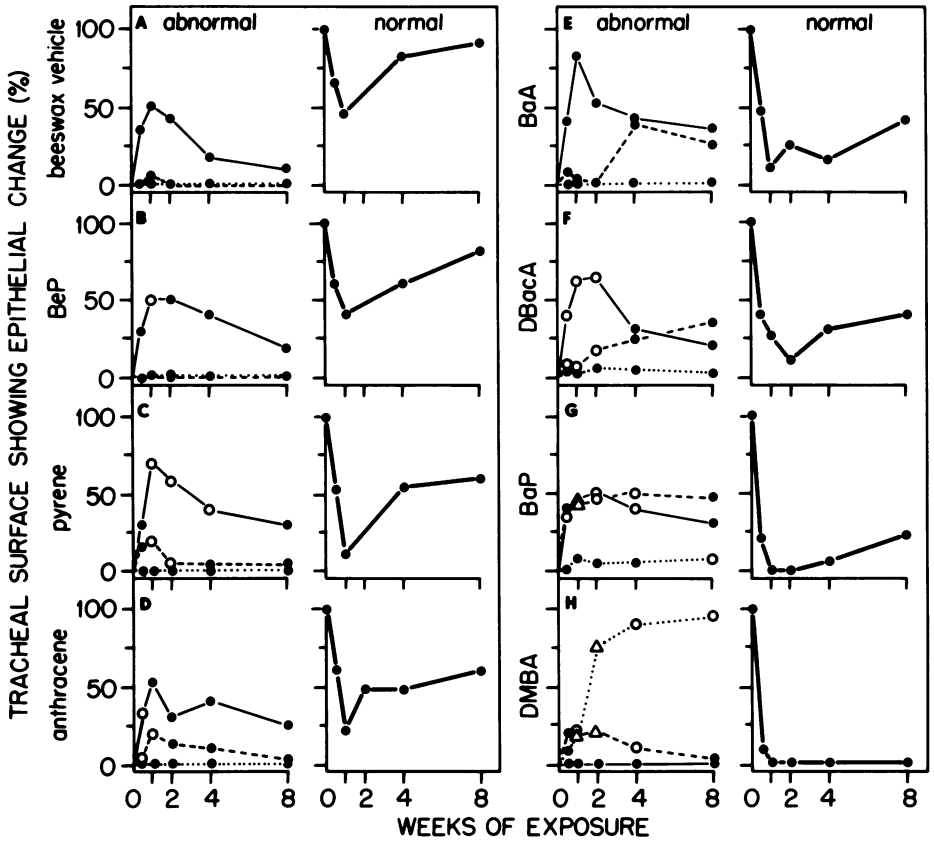

FIGURE 7. Extent and type of epithelial changes induced by various polycyclic hydrocarbons. The estimated percent tracheal surface area occupied by various epithelial morphologies is shown: (—) normal appearing epithelium; (—) hyperplasia; (--) transitional epithelium; (. . .) squamous metaplasia. Values are based on six tracheas per time point. Each trachea received 1 mg of PCH incorporated in beeswax. Control tracheas received pellets made of beeswax only. The intensity of the epithelial change is indicated as (●) mild; (○) moderate; (Δ) severe. From Topping et al. (*38*).

weak or noncarcinogenic related substances tend to induce a lesser reaction.

Tumor Induction and Cancer Morphogenesis

The advantage of the tracheal transplant model, i.e., knowing the precise quantity of carcinogen delivered to the tracheal mucosa, the exact period of time during which the mucosa is exposed to the carcinogenic agent, and the uniformity of exposure of the whole organ, have been exploited in several carcinogenesis studies. Using rat tracheal transplants continuously exposed to varying amounts of benzo(a)pyrene or 7,12-dimethylbenz(a)-anthracene in beeswax pellets, investigators in our laboratory have detected a clear dose-dependency in the tumor induction capacities of these PAHs (*41*,*42*) (Table 1). Although more than 90% of the carcinogen is released in 4 weeks from beeswax pellets containing less than 200 μg DMBA, a clear decrease in the number of induced tumors was observed when pellets were removed after 4 weeks of exposure (*43*). Only 20% of the tracheal transplants presented carcinomas, as opposed to approximately 60% when the DMBA containing pellet was left in for the whole duration of the experiment. These results are very similar to those obtained after a 4-week exposure to 200 μg DMBA-containing pellets, followed by a continuous exposure to 100 μg TPA. In this latter case, in which a two-stage carcinogenesis protocol, similar to the one employed in skin carcinogenesis studies (*44*) was employed, the percentage of induced neoplasm was 70 (*45*). From these experiments, it became obvious that the total time of exposure to PAH was at least as important as the total dose employed, and that a promotion-like effect elicited by either very small amounts of PAHs or by known promoters such as TPA or putative promoters like asbestos (*46*) is a probable pathogenic mechanism in respiratory carcinogenesis.

Using similar experimental conditions, we have extensively studied the morphogenesis of tracheal epithelial neoplasia, a detailed account of which is outside the scope of this chapter. The reader is referred to several recent reports on this subject (*43*,*47*). Briefly, the most interesting findings have been the reversibility of the early generalized toxic epithelial alterations induced by PAH (*47*) (Fig. 8), and the later appearance of focal

Table 1. Tumor development induced by BaP and DMBA in tracheal transplants.

PAH	PAH dose, μg	Total tumors/number of tracheal transplants
BaP[a]	45	0/10
	300	1/14
	900	2/12
	1250	7/13
	1740	15/10
	2160	18/12
	2490	40/52
DMBA[b]	10	0/24
	40	0/24
	115	7/24
	210	23/36
	325	11/11
	1240	12/12

[a] From Nettesheim et al. (*42*); includes all types of epithelial neoplasms.
[b] From Griesemer et al. (*41*); includes only carcinomas.

metaplastic–dysplastic lesions of varying degrees of atypia which usually precede the appearance of neoplasms (Fig. 9) (*43*). The number and degree of severity of these lesions also seems to be dose dependent. Of particular interest was the fact that many of the focal lesions regress after they reach a peak incidence, thus demonstrating that these lesions are not obligatory preneoplastic alterations.

Other studies have demonstrated a continuum of changes from the mildest to the more severe preneoplastic alterations. Quantitative techniques demonstrated increasing nucleus-cytoplasm ratios, ^{3}H-thymidine labeling indices and number of dark epithelial cells, indicating increasing degrees of cell proliferation and altered cellular maturation (*31*).

Other studies employing rat tracheal transplants have demonstrated the carcinogenic and promoting effects of asbestos (*39,46*), nickel subsulfide (*40*) and chromium carbonyl (*10*). The carcinogenic effects of PAH have also been studied in hamsters (*5,11,12*), mice (*5,16*) and dogs (*13–15*).

Repopulation of Tracheal Transplants with Isolated Cell Populations

It is often difficult to study the growth and differentiation of cultured or isolated normal or preneoplastic epithelial cells when transferred into an *in vivo* situation. Following subcutaneous or intramuscular inoculation they frequently do not survive and/or proliferate, thus making it difficult to retrieve them for morphologic evaluation. In order to be able to study sequential morphologic changes occurring *in vivo* in multiple aliquots derived from the same original cell population, we developed a technique which involves the "*in vivo* culture" of normal or preneoplastic cells on de-epithelialized tracheal stroma. The de-epithelialized stroma provides those factors required for the survival and differentiation of normal and preneoplastic epithelial

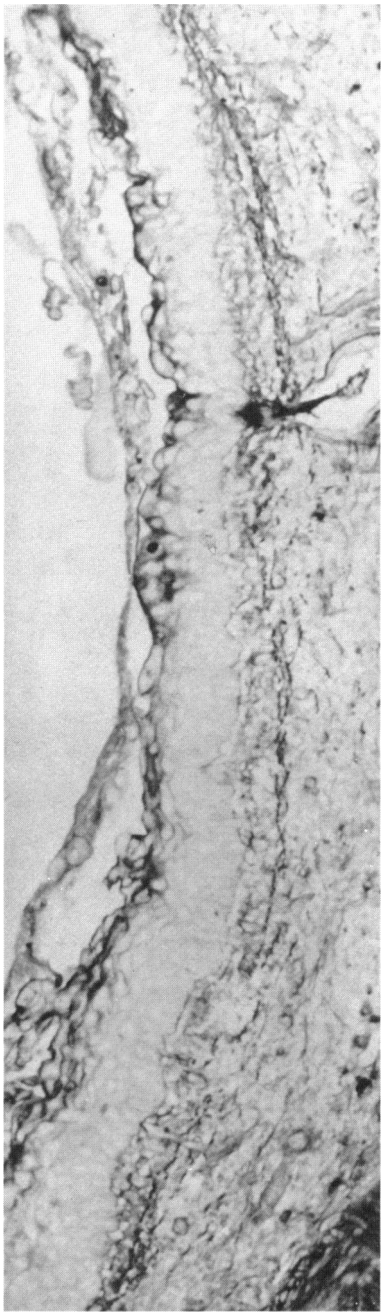

FIGURE 8. Generalized stratified metaplasia, after 2 weeks exposure to 200 μg DMBA. The surface cells contain Alcian Blue-positive material, indicative of the presence of acid mucosubstances. (PAS–Alcian Blue, pH 2.6, × 240.)

cells which are apparently lacking in the intramuscular or subcutaneous environment. The procedures have been published in detail (*36,48*). Briefly, fresh suspensions of normal respiratory tract epithelium or cultured epithelial cells originating from tracheas previously exposed to PAH *in vivo* are inoculated into the lumina of de-epithelialized tracheas. De-epithelialization is achieved by enzyme digestion (*36*) or by devitalization of

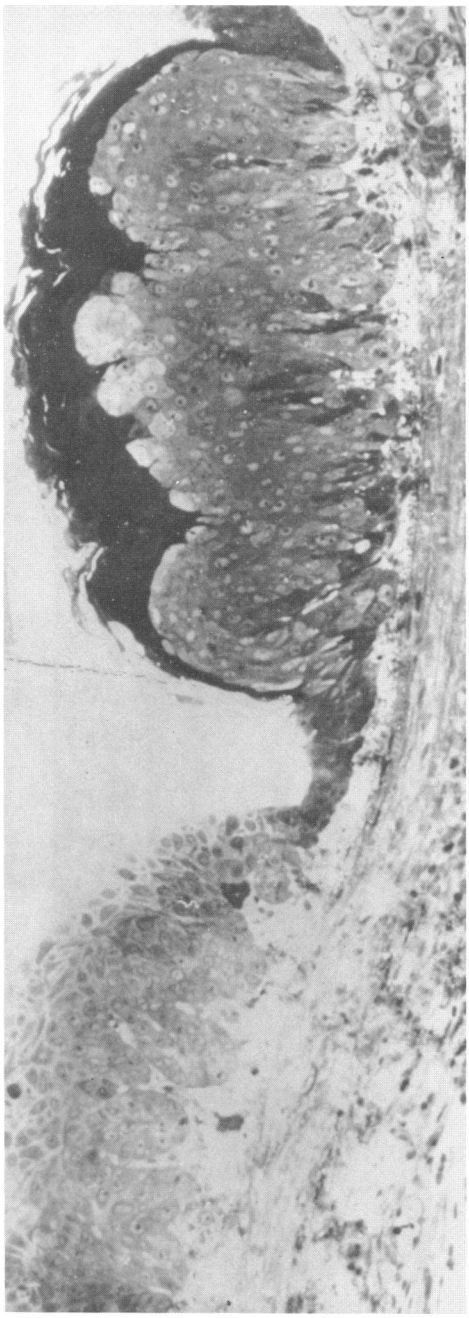

FIGURE 9. Focal atypical lesions observed after 24 weeks exposure to 200 μg DMBA. (Toluidine blue, Epon, × 300.)

the whole organ by repeated freeze-thawing (*48*). The tracheas are then transplanted into the dorsal subcutaneous tissue of host rats or nude mice. Immunocompetent hosts (rats) can be used for the growth of normal rat tracheal epithelial cells. Immunodeficient hosts (e.g., irradiated nude mice or thymectomized, irradiated rats) are generally required for long-term maintenance of preneoplastic and neoplastic cell lines. In the latter case, if immunocompetent hosts are used, the established tracheal lining rejected within 1 to 4 weeks of cell inoculation and implantation. Once inoculated tracheas are implanted into suitable hosts, approximately 2 to 3 weeks are required for the complete regeneration of a tracheal epithelium. When normal epithelial cells have been inoculated, mucociliary epithelium with occasional areas of simple or stratified squamous epithelium is seen. Inoculated preneoplastic cell lines give rise to a relatively homogeneous epithelia of either an atrophic, cuboidal, or stratified squamous nature, exhibiting various degrees of atypia (Fig. 10). After variable lengths of time during which the epithelium remains remarkably stable, some of the preneoplastic cell lines give rise *in vivo* to cell subpopulations which invade the tracheal wall. Tracheas inoculated with neoplastic cell lines are rapidly repopulated with what initially appears to be a stratified squamous epithelium exhibiting moderate to marked atypia. Within 1 to 4 weeks, one sees a rapid disorganization of this epithelium with marked cell proliferation and invasion of the transplant wall.

In general, the epithelial morphologies observed in these studies are highly reminiscent of various neoplastic and preneoplastic lesions observed following exposure of intact rat tracheas to PAH. This "*in vivo* culture system" appears to be well suited to the study of the growth and differentiation characteristics of carcinogen-altered or preneoplastic cell populations. This is otherwise not readily accomplished, since nonneoplastic cells when inoculated subcutaneously or intramuscularly either do not survive or do not proliferate and are thus often difficult to retrieve for morphologic evaluation.

This *in vivo* culture system has also been further developed for purposes of investigating differences in the stromal requirements, for growth and differentiation *in vivo*, or normal and PAH altered cell populations (*36*). For normal tracheal epithelial cell survival and maintenance of mucociliary differentiation *in vivo*, an intact but not necessarily viable stroma (tracheal, intestinal, esophageal, or bladder) is required. Preneoplastic and neoplastic cells will survive and differentiate *in vivo* on all substrates including, for example, dacron mesh which will not support normal cell growth and differentiation.

Repopulation of Tracheal Transplants with Human Epithelial Cells

Using the same repopulation techniques, it is possible to reconstitute a normal human respiratory epithelium in previously de-epithelialized rat tracheas transplanted sc in nude mice (*14*). Using cells derived directly from tissues obtained at autopsy, we have been able to establish up to 10 reconstituted tracheal transplants from each stillborn fetus. Within 2 to 4 weeks of cell inoculation and transplantation, these tracheas are fully repopulated with a normal appearing human respiratory epithelium. This epithelium is enzymatically harvested and inoculated into 30 to 50 new de-epithelialized rat tracheas and transplanted subcutaneously into a

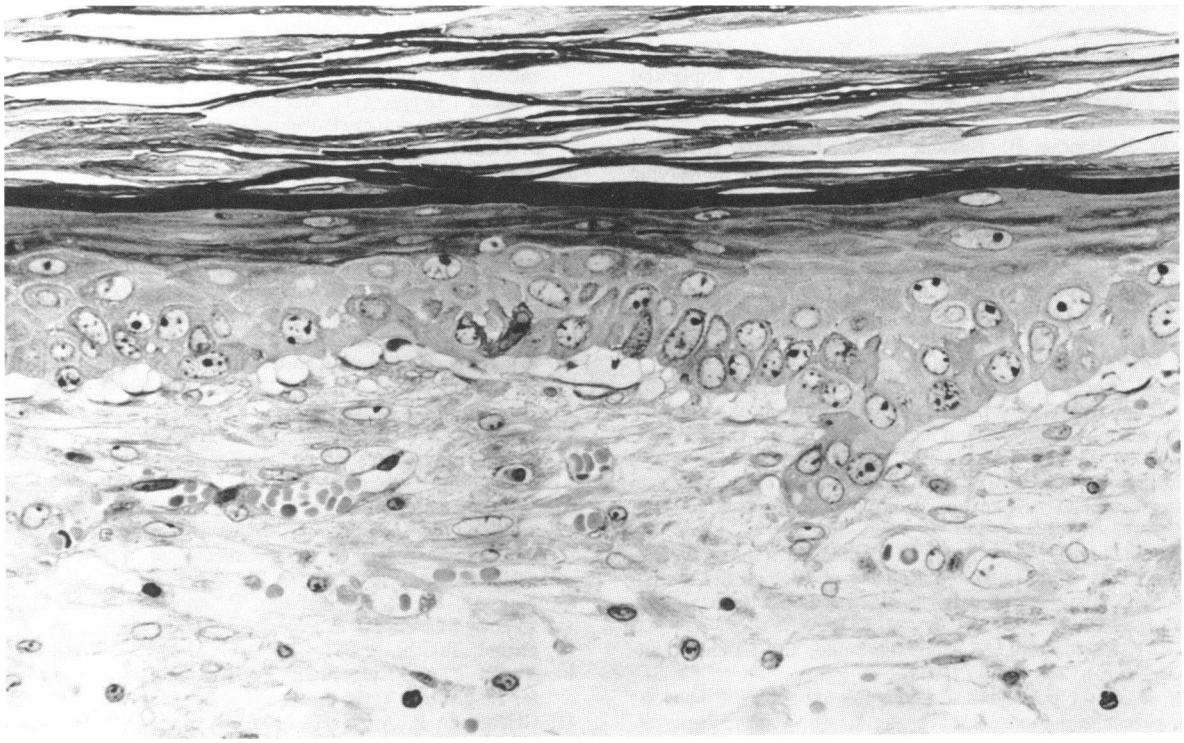

FIGURE 10. Reconstituted stratified squamous epithelium with mild atypia, as seen three weeks after inoculation of a "preneoplastic" cell line into denuded tracheas. (Toluidine blue, Epon, × 380.)

new series of host animals. In this way, with each sequential *in vivo* passage of human epithelial cells a population amplification factor of 3 to 5 is achieved. This procedure can be repeated at least four times (*49*). Preliminary experiments involving exposure of reconstituted human respiratory epithelium to DMBA suggest that studies similar to those carried out with exposed rat tracheal epithelium can also be carried out with human respiratory epithelium (*50*).

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REFERENCES

1. Nettesheim, P., and Griesemer, R. A. Experimental models for studies of respiratory tract carcinogenesis. In: Pathogenesis and Therapy of Lung Cancer (C. Harris, Ed.), Lung Biology in Health and Disease (C. Lenfant, Ed.), Vol. 10, Marcel Dekker Inc., New York, 1978, pp. 75–188.
2. Nettesheim, P., Klein-Szanto, A. J. P., Marchok, A. C., Steele, V. E., Terzaghi, M., and Topping, D. C. Studies of neoplastic development in respiratory tract epithelium. Arch. Pathol. Lab. Med. 105: 1–10 (1981).
3. Nettesheim, P., and Schreiber, H. Advances in experimental lung cancer research. In: Handbuch der Allgemeinen Pathologie (H. W. Altmann, F. Büchner and H. Cottier, Eds.) Vol. 6. Springer-Verlag, Berlin, 1975, pp. 603–691.
4. Griesemer, R. A., Kendrick, J., and Nettesheim, P. Tracheal grafts. In: Experimental Lung Cancer: Carcinogenesis and Bioassays (E. Karbe and J. F. Park, Eds.), Springer-Verlag, Berlin, 1974, pp. 539–547.
5. Kendrick, J., Nettesheim, P., and Hammons, A. S. Tumor induction in tracheal grafts: a new experimental model for respiratory carcinogenesis studies. J. Natl. Cancer Inst. 52: 1317–1325 (1974).
6. Crocker, T. T., and Nielsen, B. I. Organ culture followed by grafting of rat tracheas exposed to hydrocarbons. Proc. Am. Assoc. Cancer Res. 7: 16 (1966).
7. Dontenwill, W. Experimentelle Untersuchungen zur Genese des Lungens-carcinoma. Arzneim. Forsch. 14: 774–780 (1964).
8. Smith, W. E. The tissue transplant technique as a means of testing materials for carcinogenic action. Cancer Res. 9: 712–723 (1949).
9. Smith, W. E., Yazdi, E., and Miller, L. Carcinogenesis in pulmonary epithelia in mice on different levels of vitamin A. Environ. Res. 5: 152–163 (1973).
10. Lane, B. P., and Mass, M. J. Carcinogenicity and cocarcinogenicity of chromium carbonyl in heterotopic tracheal grafts. Cancer Res. 37: 1476–1479 (1977).
11. Craighead, J. E., and Mossman, B. T. Carcinoma induction by 3-methylcholanthrene in hamster tracheal tissue implanted in syngeneic animals. Prog. Exptl. Tumor Res. 24: 48–60 (1979).
12. Mossman, B. T., and Craighead, J. E. Induction of neoplasms in hamster tracheal grafts with 3-methylcholanthrene-coated lycra fibers. Cancer Res. 38: 3717–3722 (1978).
13. Kobayashi, N., Kanisawa, M., Okamoto, T., Okita, M., and Katsuki, H. Sequential cytologic study of the development of squamous cell carcinoma induced in subcutaneously implanted bronchial autograft of dog. Acta Cytol. 22: 99–104 (1978).
14. Kobayashi, N., Okita, M., Yarita, T., Hanzawa, S., Okamoto, T., and Katsuki, H. A method for experimental induction of bronchogenic carcinoma in subcutaneously implanted bronchial

autograft in dogs, J. Thorac. Cardiovasc. Surg. 75: 434–442 (1978).
15. Kobayashi, N., Okamoto, T., Yarita, T., Tanaka, T., Okita, M., and Katsuki, H. Cancer induction in subcutaneously implanted bronchial autografts of a dog by 3-methylcholanthrene. Gann 67: 611–615 (1976).
16. Ebenstein, P., Kinder, B., Bankole, D. O., Richards, F. F., and Armstrong, M. Y. K. Expression of endogenous xenotropic retrovirus by methylcholanthrene-induced squamous cell carcinoma of the mouse respiratory tract. J. Exptl. Med. 150: 1567–1570 (1979).
17. Pal, B. C., Topping, D. C., Griesemer, R. A., Nelson, F. R., and Nettesheim, P. Development of a system for controlled release of benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, and phorbol ester for tumor induction in heterotopic tracheal grafts. Cancer Res. 38: 1376–1383 (1978).
18. Shors, E. C., Fu, P. C., Matsumura, K., Cohen, A., and Benfield, J. R. Sustained release of benzo(a)pyrene from silicone polymer into the tracheobronchial tree of hamsters and dogs. Cancer Res. 40: 2288–2294 (1980).
19. Higuchi, T. Rate of release of medicaments from ointment bases containing drugs in suspension. J. Pharm. Sci. 52: 1145–1150 (1963).
20. Baker, R. W., and Londsdale, H. K. Controlled release: mechanisms and rates. In: Controlled Release of Biologically Active Agents (A. C. Tanguary and R. E. Lacy, Eds.), Plenum Press, NY, 1973, pp. 15–71.
21. Fieser, L. F. Cholesterol, Δ5-cholesten-3-one, and Δ4-cholesten-3-one. Org. Syn. Coll. 4: 195 (1963).
22. Terzaghi, M., and Nettesheim, P. Dynamics of neoplastic development in carcinogen-exposed tracheal mucosa. Cancer Res. 39: 4003–4010 (1979).
23. Smith, L. L., Smart, V. B., and Ansari, G. A. S. Mutagenic cholesterol preparations. Mutat. Res. 68: 23–30 (1979).
24. Clark, J. N., and Marchok, A. C. Characterization of mucin isolated from rat tracheal transplants. Biochim. Biophys. Acta 588: 357–367 (1979).
25. Clark, J. N., Klein-Szanto, A. J. P., Pine, A. H., Stephenson, K. B., and Marchok, A. C. Reestablishment of a mucociliary epithelium in tracheal organ cultures exposed to retinyl acetate: a biochemical and morphometric study. Eur. J. Cell Biol. 21: 261–268 (1980).
26. Marchok, A. C., Clark, J. N., and Klein-Szanto, A. J. P. Modulation of growth, differentiation, and mucous glycoprotein synthesis by retinyl acetate in cloned carcinoma cell lines. J. Natl. Cancer Inst. 66: 1165–1174 (1981).
27. Schreiber, H. Cytopathology in pathogenesis and therapy of lung cancer. In: Lung Biology in Health and Disease (C. Lenfant, Ed.), Vol. 10. Marcel Dekker, New York, 1978, p. 521.
28. Klein-Szanto, A. J. P., Topping, D. C., Heckman, C. A., and Nettesheim, P. Ultrastructural characteristics of carcinogen-induced dysplastic changes in tracheal epithelium. Am. J. Pathol. 98: 83–100 (1980).
29. Klein-Szanto, A. J. P., Topping, D. C., Heckman, C. A., and Nettesheim, P. Ultrastructural characteristics of carcinogen-induced non-dysplastic changes in tracheal epithelium. Am J. Pathol. 98: 61–82 (1980).
30. Klein-Szanto, A. J. P., Nettesheim, P., Pine, A., and Martin, D. Distribution and ultrastructural characteristics of dark cells in squamous metaplasias of the respiratory tract epithelium. Am. J. Pathol. 103: 263–270 (1981).
31. Klein-Szanto, A. J. P., Nettesheim, P., Topping, D. C., and Olson, A. C. Quantitative analysis of disturbed cell maturation in dysplastic lesions of the respiratory tract epithelium. Carcinogenesis 1: 1007–1016 (1980).
32. Knudtson, K. P. A method of study of squamous metaplasia, atypical change, an early bronchogenic carcinoma of the bronchial mucosa in smokers and nonsmokers. Cancer 9: 84–85 (1956).
33. Watt, J., Gregory, I., and Stell, P. M. A simple method for the post-mortem demonstration of squamous metaplasia in the larynx. J. Pathol. 104: 149–154 (1970).
34. Watt, J., Gregory, I., and Stell, P. M. An alcian-blue phloxine method for the gross demonstrations of squamous metaplasia in the larynx. J. Pathol. 116: 31–36 (1975).
35. Marchok, A. C., Rhoton, J. C., and Nettesheim, P. *In vitro* development of oncogenicity in cell lines established from tracheal epithelium preexposed *in vivo* to 7,12-dimethylbenz(a)anthracene. Cancer Res. 38: 2030–2037 (1978).
36. Terzaghi, M., Nettesheim, P., and Williams, M. L. Repopulation of denuded tracheal grafts with normal, preneoplastic and neoplastic epithelial cell populations. Cancer Res. 38: 4546–4553 (1978).
37. Terzaghi, M., Nettesheim, P., and Riester, L. Effect of carcinogen dose on dynamics of neoplastic development on rat tracheal epithelium. Cancer Res. 42: 4511–4518 (1982).
38. Topping, D. C., Pal, B. C., Martin, D. H., Nelson, F. R., and Nettesheim, P. Pathologic changes induced in respiratory tract mucosa by polycyclic hydrocarbons of differing carcinogenic activity. Am. J. Pathol. 93: 311–324 (1978).
39. Topping, D. C., Nettesheim, P., and Martin, D. H. Toxic and tumorigenic effects of asbestos on tracheal mucosa. J. Environ. Pathol. Toxicol. 3: 261–275 (1980).
40. Yarita, T., and Nettesheim, P. Carcinogenicity of nickel subsulfide for respiratory tract mucosa. Cancer Res. 38: 3140–3145 (1978).
41. Griesemer, R. A., Nettesheim, P., Martin, D. H., and Caton, J. E., Jr. Quantitative exposure of grafted rat tracheas to 7,12-dimethylbenz(a)anthracene. Cancer Res. 37: 1266–1271 (1977).
42. Nettesheim, P., Griesemer, R. A., Martin, D. H., and Caton, J. E., Jr. Induction of preneoplastic and neoplastic lesions in grafted rat tracheas continuously exposed to benzo(a)pyrene. Cancer Res. 37: 1272–1278 (1977).
43. Topping, D. C., Griesemer, R. A., and Nettesheim, P. Development and fate of focal epithelial lesions in tracheal mucosa following carcinogen exposure. Cancer Res. 39: 4829–4837 (1978).
44. Boutwell, R. K. Function and mechanisms of promoters of carcinogenesis. CRC Crit. Rev. Toxicol. 2: 419–443 (1974).
45. Topping, D. C., and Nettesheim, P. Promotion-like enhancement of tracheal carcinogenesis in rats by TPA (12-O-tetradecanoylphorbol-13-acetate). Cancer Res. 40: 4352–4355 (1980).
46. Topping, D. C., and Nettesheim, P. Two-stage carcinogenesis studies with asbestos in Fischer 344 rats. J. Natl. Cancer Inst. 65: 627–630 (1980).
47. Topping, D. C., Griesemer, R. A., and Nettesheim, P. Quantitative assessment of generalized epithelial changes in tracheal mucosa following exposure to 7,12-dimethylbenz(a)anthracene. Cancer Res. 39: 4823–4828 (1979).
48. Terzaghi, M., and Klein-Szanto, A. J. P. Differentiation of normal and cultured preneoplastic tracheal epithelial cells in rats: importance of epithelial-mesenchymal interactions. J. Natl. Cancer Inst. 65: 1039–1048 (1980).
49. Klein-Szanto, A. J. P., and Terzaghi, M. Continuous propagation of normal human respiratory epithelial cells using an *in vivo* "culture system"—an experimental model for the study of human disease. Lab. Invest. 44: 34A (1981).
50. Terzaghi, M., Klein-Szanto, A. J. P., and Shiba, M. An in vivo-in vitro model for quantitative studies on the dynamics of neoplastic development in human respiratory epithelium. Proc. Am. Assoc. Cancer Res. 22: 135 (1981).